L Number	Hits	Search Text	DB	Time stamp
3	6	XUE-RU	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 17:49
-	11260	sun.in.	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 16:40
-	116	sun.in. and kidney$15	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 16:40
-	11	(sun.in. and kidney$15) and urine	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 17:48
-	70	kidney ADJ specific	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 16:53
-	2	(kidney ADJ specific) and uromodulin	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 16:54
-	42	uromodulin	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 16:57
-	19	uromodulin and kidney	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 17:08
-	9	kidney$10 ADJ specific ADJ promoter	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 17:14
-	4	(kidney ADJ specific) and (apical or basolateral)	USPAT; US-PGPUB; EPO; JPO; DERWENT; USOCR	2002/04/23 17:15

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     (FILE 'HOME' ENTERED AT 17:17:41 ON 23 APR 2002)

     FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF'
     ENTERED AT 17:17:50 ON 23 APR 2002
L1            16 S KIDNEY SPECIFIC PROMOTER
L2             7 DUP REM L1 (9 DUPLICATES REMOVED)
L3             7 SORT L2 PY
L4             0 S APICAL SURFACE MEMBRANE TARGET? SEQUENCE?
L5            58 S (BASOLATRAL SURFACE MEMBRANE) OR (APICAL SURFACE MEMBRANE)
L6             0 S L5 AND (TARGE? SEQ?)
L7            28 DUP REM L5 (30 DUPLICATES REMOVED)
L8            28 SORT L7 PY
L9             0 S L7 AND PROMOTER
L10       143789 S PIPLC OR PHOSPHATIDYLINOSITOL? OR (PHOSPHOLIPASE C)
L11         5821 S PHOSPHATIDYLINOSITOL?-SPECIFIC PHOSPHOLIPASE C
L12         5820 S PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C
L13         1549 S L12 AND (DNA OR NUCLEIC OR SEQUENCE OR GENE)
L14           95 S L13 AND KIDNEY
L15           43 DUP REM L14 (52 DUPLICATES REMOVED)
L16           43 FOCUS L15 1-
L17            0 S L15 AND PROMOTER
                E SUN TUNG?/AU
L18          191 S E2
L19           12 S L18 AND KIDNEY
L20            8 DUP REM L19 (4 DUPLICATES REMOVED)
L21            8 SORT L20 PY
L22            1 S UROMODULIN PROMOTER

=> d 122 all

L22  ANSWER 1 OF 1  CAPLUS  COPYRIGHT 2002 ACS
AN   2000:351690  CAPLUS
DN   133:13401
TI   Transgenic animals as bioreactors for production of protein in urine by
     kidney-specific expression using the uromodulin gene promoter
IN   Wu, Xue-Ru; Sun, Tung-Tien
PA   New York University, USA
SO   PCT Int. Appl., 55 pp.
     CODEN: PIXXD2
DT   Patent
LA   English
IC   ICM  C12P021-00
     ICS  A01K067-00; C07H021-02; C07H021-04; C12N015-00
CC   3-2 (Biochemical Genetics)
     Section cross-reference(s): 13, 16
FAN.CNT 1
     PATENT NO.        KIND  DATE           APPLICATION NO.   DATE
     ---------------   ----  --------       ---------------   --------
PI   WO 2000029608      A1   20000525       WO 1999-US26870   19991112
         W:  AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU,
             CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL,
             IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA,
             MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI,
             SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, AM, AZ,
             BY, KG, KZ, MD, RU, TJ, TM
         RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE,
             DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF,
             CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG
     EP 1135518         A1   20010926       EP 1999-958952    19991112
         R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
             IE, SI, LT, LV, FI, RO
PRAI US 1998-108195P    P    19981113
     US 1999-142925P    P    19990709
     WO 1999-US26870    W    19991112
AB   The invention relates to recombinant DNA constructs, a method for
     producing a recombinant biol. active protein in vivo in the urine of a
     non-human mammal using a kidney-specific promoter, such as the
     uromodulin promoter, and the transgenic non-human
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mammals that serve as urine-based bioreactors for protein prodn. The recombinant DNA construct may also contain a secretion signal sequence operably linked to the heterologous gene. The method for producing a recombinant biol. active protein in vivo in the urine of a non-human mammal comprises the steps of introducing the recombinant DNA construct into a fertilized embryo to produce a transgenic non-human mammals expressing and secreting the protein in the urine, and collecting the urine to recover the protein. The **uromodulin promoter** is preferably of mouse, cattle, or rat, and the transgenic non-human mammal is goat, cow, sheep, pig, or horse. The nucleotide sequences of the mouse and goat uromodulin gene promoter region were obtained. Recombinant prodn. of human growth hormone in the urine of transgenic mouse using the **uromodulin promoter** is described. (no data).

ST mouse goat uromodulin gene promoter sequence; urine protein recombinant prodn **uromodulin promoter**

IT Promoter (genetic element)
RL: BOC (Biological occurrence); BSU (Biological study, unclassified); BUU (Biological use, unclassified); PRP (Properties); BIOL (Biological study); OCCU (Occurrence); USES (Uses)
(for uromodulin gene of mouse, cattle, and rat; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Tamm-Horsfall glycoprotein
RL: BSU (Biological study, unclassified); BIOL (Biological study)
(gene for, promoter of; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Cattle
Goat
Horse (Equus caballus)
Mammal (Mammalia)
Sheep
Swine
(host animal for recombinant protein prodn.; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Promoter (genetic element)
RL: BOC (Biological occurrence); BSU (Biological study, unclassified); BUU (Biological use, unclassified); PRP (Properties); BIOL (Biological study); OCCU (Occurrence); USES (Uses)
(kidney-specific; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT DNA sequences
(of mouse uromodulin gene promoter; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Genetic element
RL: BUU (Biological use, unclassified); BIOL (Biological study); USES (Uses)
(signal sequence, linked to transgene; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Bioreactors
Fermentation
Genetic vectors
Molecular cloning
Urine
(transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Transgene
RL: BPR (Biological process); BSU (Biological study, unclassified); BUU (Biological use, unclassified); BIOL (Biological study); PROC (Process); USES (Uses)
(transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT Cattle
Goat
Mouse

Rat
(**uromodulin promoter** source; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT 271754-52-0
RL: BOC (Biological occurrence); BSU (Biological study, unclassified); BUU (Biological use, unclassified); PRP (Properties); BIOL (Biological study); OCCU (Occurrence); USES (Uses)
(nucleotide sequence; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using uromodulin gene promoter)

IT 271762-90-4, 1: PN: WO0029608 PAGE: 16 unclaimed DNA 271762-91-5, 2: PN: WO0029608 PAGE: 16 unclaimed DNA 271762-92-6, 3: PN: WO0029608 PAGE: 16 unclaimed DNA 271762-93-7, 4: PN: WO0029608 PAGE: 16 unclaimed DNA 271762-94-8, 6: PN: WO0029608 PAGE: 17 unclaimed DNA 271762-95-9, 7: PN: WO0029608 PAGE: 17 unclaimed DNA 271762-96-0, 10: PN: WO0029608 PAGE: 18 unclaimed DNA 271762-97-1, 11: PN: WO0029608 PAGE: 18 unclaimed DNA 271762-98-2, 12: PN: WO0029608 PAGE: 20 unclaimed DNA 271762-99-3, 13: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-00-9, 14: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-01-0, 15: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-02-1, 16: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-03-2, 17: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-04-3, 18: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-05-4, 19: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-06-5, 20: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-07-6, 21: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-08-7, 22: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-09-8, 23: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-10-1, 24: PN: WO0029608 PAGE: 20 unclaimed DNA 271763-11-2, 25: PN: WO0029608 PAGE: 22 unclaimed DNA 271763-12-3, 27: PN: WO0029608 PAGE: 23 unclaimed DNA 271763-13-4, 28: PN: WO0029608 PAGE: 23 unclaimed DNA 271763-14-5, 29: PN: WO0029608 PAGE: 24 unclaimed DNA 271763-15-6, 30: PN: WO0029608 PAGE: 24 unclaimed DNA 271763-16-7, 31: PN: WO0029608 PAGE: 24 unclaimed DNA 271763-17-8, 32: PN: WO0029608 PAGE: 24 unclaimed DNA 271763-18-9, 33: PN: WO0029608 PAGE: 24 unclaimed DNA 271763-19-0, 34: PN: WO0029608 FIG: 9 unclaimed DNA
RL: PRP (Properties)
(unclaimed nucleotide sequence; transgenic animals as bioreactors for prodn. of protein in urine by kidney-specific expression using the uromodulin gene promoter)

RE.CNT 5 THERE ARE 5 CITED REFERENCES AVAILABLE FOR THIS RECORD
RE
(1) Jeanpierre; Cytogetic and Cell Genetics V62, P185 CAPLUS
(2) Kerr; Nature Biotechnology V16, P75 CAPLUS
(3) Su; The Journal of Immunology V158, P3449 CAPLUS
(4) Yu; Gene Expression V4, P63 CAPLUS
(5) Zhong-Ting; Cancer Research V59, P3512

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(FILE 'HOME' ENTERED AT 17:17:41 ON 23 APR 2002)

FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF' ENTERED AT 17:17:50 ON 23 APR 2002

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L1            16 S KIDNEY SPECIFIC PROMOTER
L2             7 DUP REM L1 (9 DUPLICATES REMOVED)
L3             7 SORT L2 PY
L4             0 S APICAL SURFACE MEMBRANE TARGET? SEQUENCE?
L5            58 S (BASOLATRAL SURFACE MEMBRANE) OR (APICAL SURFACE MEMBRANE)
L6             0 S L5 AND (TARGE? SEQ?)
L7            28 DUP REM L5 (30 DUPLICATES REMOVED)
L8            28 SORT L7 PY
L9             0 S L7 AND PROMOTER
L10       143789 S PIPLC OR PHOSPHATIDYLINOSITOL? OR (PHOSPHOLIPASE C)
L11         5821 S PHOSPHATIDYLINOSITOL?-SPECIFIC PHOSPHOLIPASE C
L12         5820 S PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE C
L13         1549 S L12 AND (DNA OR NUCLEIC OR SEQUENCE OR GENE)
L14           95 S L13 AND KIDNEY
L15           43 DUP REM L14 (52 DUPLICATES REMOVED)
L16           43 FOCUS L15 1-
L17            0 S L15 AND PROMOTER
                E SUN TUNG?/AU
L18          191 S E2
L19           12 S L18 AND KIDNEY
L20            8 DUP REM L19 (4 DUPLICATES REMOVED)
L21            8 SORT L20 PY
```

=> d an ti so au ab l16 6

L16 ANSWER 6 OF 43 MEDLINE
AN 91065873 MEDLINE
TI Uromodulin (Tamm-Horsfall glycoprotein/uromucoid) is a phosphatidylinositol-linked membrane protein.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1990 Dec 5) 265 (34) 20784-9. Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Rindler M J; Naik S S; Li N; Hoops T C; Peraldi M N
AB Uromodulin, originally identified as an immunosuppressive glycoprotein in the urine of pregnant women, has been previously shown to be identical to human Tamm-Horsfall glycoprotein (THP). THP is synthesized by the **kidney** and localizes to the renal thick ascending limb and early distal tubule. It is released into the urine in large quantities and thus represents a potential candidate for a protein secreted in a polarized fashion from the apical plasma membrane of epithelial cells in vivo. After introduction of the full-length cDNA encoding uromodulin/THP into HeLa, Caco-2, and Madin-Darby canine **kidney** cells by transfection, however, the expressed glycoprotein was almost exclusively cell-associated, as determined by immunoprecipitation after radioactive labeling of the cells. By immunofluorescence, THP was localized to the plasma membranes of transfected cells. In transfected cell extracts, THP also remained primarily in the detergent phase in a Triton X-114 partitioning assay, indicating that it has a hydrophobic character, in contrast to its behavior after isolation from human urine. Triton X-114 detergent-associated THP was redistributed to the aqueous phase after treatment of cell extracts with **phosphatidylinositol-specific phospholipase C**. Treatment of intact transfected HeLa cells with **phosphatidylinositol-specific phospholipase C** also resulted in the release of THP into the medium, suggesting that it is a glycosylphosphatidylinositol (GPI)-linked membrane protein. Similar to other known GPI-linked proteins, uromodulin/THP contains a stretch of 16 hydrophobic amino acids at its extreme carboxyl-terminus which could function as a GPI addition signal and was shown to label with [3H]ethanolamine. The results indicate that THP is a member of this class of lipid-linked membrane proteins and is released into the urine after the loss of its hydrophobic anchor, probably by the action of a phospholipase or protease.

=> d an ti so au ab pi l21 8

L21 ANSWER 8 OF 8 CAPLUS COPYRIGHT 2002 ACS
AN 2001:47690 CAPLUS
DN 134:176094
TI Urothelial function reconsidered: a role in urinary protein secretion
SO Proceedings of the National Academy of Sciences of the United States of America (2001), 98(1), 154-158
CODEN: PNASA6; ISSN: 0027-8424
AU Deng, Fang-Ming; Ding, Mingxiao; Lavker, Robert M.; **Sun, Tung-Tien**
AB Mammalian bladder epithelium functions as an effective permeability barrier. We demonstrate here that this epithelium can also function as a secretory tissue directly involved in modifying urinary protein compn. Our data indicate that normal bovine urothelium synthesizes, as its major differentiation products, two well-known proteases: tissue-type plasminogen activator and urokinase, as well as a serine protease inhibitor, PP5. Moreover, we demonstrate that the urothelium secretes these proteins in a polarized fashion into the urine via a cAMP- and calcium-regulated pathway. Urinary plasminogen activators of ruminants are therefore urothelium derived rather then **kidney** derived as in some other species; this heterogeneity may have evolved in response to different physiol. or dietary factors. In conjunction with our recent finding that transgenic mouse urothelium can secrete ectopically expressed human growth hormone into the urine, our data establish that normal mammalian urothelium can function not only as a permeability barrier but also as a secretor of urinary proteins that can play physiol. or pathol. roles in the urinary tract.

=> d an ti so au ab pi l21 7

L21 ANSWER 7 OF 8 CAPLUS COPYRIGHT 2002 ACS
AN 2000:351690 CAPLUS
DN 133:13401
TI Transgenic animals as bioreactors for production of protein in urine by **kidney**-specific expression using the uromodulin gene promoter
SO PCT Int. Appl., 55 pp.
CODEN: PIXXD2
IN Wu, Xue-Ru; **Sun, Tung-Tien**
AB The invention relates to recombinant DNA constructs, a method for producing a recombinant biol. active protein in vivo in the urine of a non-human mammal using a **kidney**-specific promoter, such as the uromodulin promoter, and the transgenic non-human mammals that serve as urine-based bioreactors for protein prodn. The recombinant DNA construct may also contain a secretion signal sequence operably linked to the heterologous gene. The method for producing a recombinant biol. active protein in vivo in the urine of a non-human mammal comprises the steps of introducing the recombinant DNA construct into a fertilized embryo to produce a transgenic non-human mammals expressing and secreting the protein in the urine, and collecting the urine to recover the protein. The uromodulin promoter is preferably of mouse, cattle, or rat, and the transgenic non-human mammal is goat, cow, sheep, pig, or horse. The nucleotide sequences of the mouse and goat uromodulin gene promoter region were obtained. Recombinant prodn. of human growth hormone in the urine of transgenic mouse using the uromodulin promoter is described. (no data).

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 2000029608	A1	20000525	WO 1999-US26870	19991112
W: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW, AM, AZ, BY, KG, KZ, MD, RU, TJ, TM				
RW: GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW, AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG				
EP 1135518	A1	20010926	EP 1999-958952	19991112
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				

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(FILE 'HOME' ENTERED AT 11:17:52 ON 26 APR 2002)

FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF'
ENTERED AT 11:18:05 ON 26 APR 2002

```
L1             0 S UROPLAKKIN?
L2           354 S UROPLAKIN?
L3            98 S L2 AND (APICAL OR BASOLATRAL OR GPI OR PIPLC)
L4            34 DUP REM L3 (64 DUPLICATES REMOVED)
L5            34 FOCUS L4 1-
L6            34 SORT L5 PY
L7            99 S L2 AND (APICAL OR BASOLATERAL OR GPI OR PIPLC)
L8            35 DUP REM L7 (64 DUPLICATES REMOVED)
L9            34 S L6 AND L8
L10            4 S L9 AND BASOLATERAL
                 E SUN T?/AU
                 E SUN TUNG?/AU
L11          191 S E2
L12           29 S L11 AND L7
L13           16 DUP REM L12 (13 DUPLICATES REMOVED)
L14           16 SORT L13 PY
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FILE 'STNGUIDE' ENTERED AT 11:40:31 ON 26 APR 2002

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L15            0 S GLYCOSYL PHOSPHATIDYLINOSITOL
L16            0 S PHOSPHATIDYLINOSITOL
L17            0 S GLYCOSYL PHOSP?
L18            0 S C-TERMINAL GLYCOSYL?
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FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF'
ENTERED AT 11:45:16 ON 26 APR 2002

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L19           84 S C-TERMINAL GLYCOSYL?
L20           26 S L19 AND PHOSPHATI?
L21            8 DUP REM L20 (18 DUPLICATES REMOVED)
L22            8 SORT L21 PY
L23            8 S  (C-TERMINAL GLYCOSYLPHOSPHATIDYLINOSITOL) AND (SIGNAL SEQUEN
L24            2 DUP REM L23 (6 DUPLICATES REMOVED)
L25         3348 S BASOLATERAL AND SIGNAL?
L26         2943 S BASOLATERAL (L) SIGNAL?
L27            0 S L26 AND (BASOLATRAL SURFACE)
L28          767 S L26 AND TARGET?
L29          272 S L28 AND SEQUENCE
L30          227 S L29 AND MEMBRANE
L31           94 DUP REM L30 (133 DUPLICATES REMOVED)
L32           94 FOCUS L31 1-
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     (FILE 'HOME' ENTERED AT 11:17:52 ON 26 APR 2002)

     FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF'
     ENTERED AT 11:18:05 ON 26 APR 2002
L1             0 S UROPLAKKIN?
L2           354 S UROPLAKIN?
L3            98 S L2 AND (APICAL OR BASOLATRAL OR GPI OR PIPLC)
L4            34 DUP REM L3 (64 DUPLICATES REMOVED)
L5            34 FOCUS L4 1-
L6            34 SORT L5 PY
L7            99 S L2 AND (APICAL OR BASOLATERAL OR GPI OR PIPLC)
L8            35 DUP REM L7 (64 DUPLICATES REMOVED)
L9            34 S L6 AND L8
L10            4 S L9 AND BASOLATERAL
                 E SUN T?/AU
                 E SUN TUNG?/AU
L11          191 S E2
L12           29 S L11 AND L7
L13           16 DUP REM L12 (13 DUPLICATES REMOVED)
L14           16 SORT L13 PY

     FILE 'STNGUIDE' ENTERED AT 11:40:31 ON 26 APR 2002
L15            0 S GLYCOSYL PHOSPHATIDYLINOSITOL
L16            0 S PHOSPHATIDYLINOSITOL
L17            0 S GLYCOSYL PHOSP?
L18            0 S C-TERMINAL GLYCOSYL?

     FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF'
     ENTERED AT 11:45:16 ON 26 APR 2002
L19           84 S C-TERMINAL GLYCOSYL?
L20           26 S L19 AND PHOSPHATI?
L21            8 DUP REM L20 (18 DUPLICATES REMOVED)
L22            8 SORT L21 PY
L23            8 S  (C-TERMINAL GLYCOSYLPHOSPHATIDYLINOSITOL) AND (SIGNAL SEQUEN
L24            2 DUP REM L23 (6 DUPLICATES REMOVED)
L25         3348 S BASOLATERAL AND SIGNAL?
L26         2943 S BASOLATERAL (L) SIGNAL?
L27            0 S L26 AND (BASOLATRAL SURFACE)
L28          767 S L26 AND TARGET?
L29          272 S L28 AND SEQUENCE
L30          227 S L29 AND MEMBRANE
L31           94 DUP REM L30 (133 DUPLICATES REMOVED)
L32           94 FOCUS L31 1-

=> d an ti so au ab pi l32 1 2 4 8 9

L32  ANSWER 1 OF 94  CAPLUS  COPYRIGHT 2002 ACS
AN   2001:267787  CAPLUS
DN   134:320967
TI   Identification of a basolateral sorting signal for the
     M3 muscarinic acetylcholine receptor in Madin-Darby canine kidney cells
SO   Journal of Biological Chemistry (2001), 276(13), 10539-10547
     CODEN: JBCHA3; ISSN: 0021-9258
AU   Nadler, Laurie S.; Kumar, Geetha; Nathanson, Neil M.
AB   Muscarinic acetylcholine receptors (mAChRs) can be differentially
     localized in polarized cells.  To identify potential sorting
     signals that mediate mAChR targeting, the authors examd.
     the sorting of mAChRs in Madin-Darby canine kidney cells, a widely used
     model system.  Expression of FLAG-tagged mAChRs in polarized Madin-Darby
     canine kidney cells demonstrated that the M2 subtype is sorted apically,
     whereas M3 is targeted basolaterally.  Expression of M2/M3
     receptor chimeras revealed that a 21-residue sequence,
     Ser271-Ser291, from the M3 third intracellular loop contains a
     basolateral sorting signal.  Substitution of
     sequences contg. the M3 sorting signal into the
     homologous regions of M2 was sufficient to confer basolateral
     localization to this apical receptor.  Sequences contg. the M3
     sorting signal also conferred basolateral
     targeting to M2 when added to either the third intracellular loop
```

or the C-terminal cytoplasmic tail. Furthermore, addn. of a **sequence** contg. the M3 **basolateral** sorting **signal** to the cytoplasmic tail of the interleukin-2 receptor .alpha.-chain caused significant **basolateral targeting** of this heterologous apical protein. The results indicate that the M3 **basolateral** sorting **signal** is dominant over apical **signals** in M2 and acts in a position-independent manner. The M3 sorting **signal** represents a novel **basolateral targeting** motif for G protein-coupled receptors.

L32 ANSWER 2 OF 94 CAPLUS COPYRIGHT 2002 ACS
AN 2001:474356 CAPLUS
DN 135:150364
TI Two distinct **signals** determine the **basolateral targeting** of AQP4 in the renal epithelial cell line MDCK
SO Molecular Biology and Physiology of Water and Solute Transport, [Proceedings of the International Conference on Molecular Biology and Physiology of Water and Solute Transport: Fundamental and Applied Aspects], 3rd, Goeteborg, Sweden, July 1-14, 2000 (2000), 159-165. Editor(s): Hohmann, Stefan; Nielsen, Soren. Publisher: Kluwer Academic/Plenum Publishers, New York, N. Y.
CODEN: 69BKXR
AU Madrid, Ricardo; Le Maout, Sophie; Barrault, Marie-Benedicte; Merot, Jean
AB MDCK cells represent a valuable cellular model system to analyze the mechanisms of aquaporin **targeting** in polarized epithelial cells. Using engineered epitope-tagged AQP4 and mutagenesis we showed that AQP4 **basolateral targeting** is dependent on 2 independent **signals** contained in the cytoplasmic C-terminal domain of the protein. Interestingly, these 2 peptidic **sequences** contain putative tyrosine-based and double leucine **signals** that have been shown to be crit. sorting determinants of basolaterally **targeted** proteins.

L32 ANSWER 4 OF 94 MEDLINE
AN 94253163 MEDLINE
TI The **basolateral targeting signal** in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus resembles a variety of intracellular **targeting** motifs related by primary **sequence** but having diverse **targeting** activities.
SO JOURNAL OF BIOLOGICAL CHEMISTRY, (1994 Jun 3) 269 (22) 15732-9.
Journal code: HIV; 2985121R. ISSN: 0021-9258.
AU Thomas D C; Roth M G
AB Using systematic site-directed mutagenesis, the **basolateral targeting signal** in the cytoplasmic domain of glycoprotein G from vesicular stomatitis virus (VSV G) has been localized to an 11-amino acid **sequence**, which contains two essential residues and a third that makes a minor contribution. A tyrosine at position 19 of the 29-residue carboxyl-terminal cytoplasmic tail is the most important residue and cannot be replaced by other aromatic amino acids, while an isoleucine at position 22, 3 residues carboxyl-terminal to this tyrosine, is also critical but can be replaced by other aliphatic residues. Additionally, an arginine at position 16 makes a minor contribution. Therefore the crucial elements of this **targeting signal** can be represented by the **sequence** Y-X-X-aliphatic. While earlier investigation has suggested similarity between **basolateral targeting** and internalization **signals**, alignment of this **sequence** with other cytoplasmic **targeting signals** suggests the existence of a broad class of homologous **targeting** motifs that direct protein delivery to a variety of cellular locations. This in turn suggests the existence of a family of homologous receptors, distributed throughout the cell, which differ in their affinity for subsets of these **targeting sequences**.

L32 ANSWER 8 OF 94 MEDLINE
AN 2001483207 MEDLINE
TI Basolateral **membrane** expression of a K+ channel, Kir 2.3, is directed by a cytoplasmic COOH-terminal domain.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF

AMERICA, (2001 Aug 28) 98 (18) 10475-80.
Journal code: PV3; 7505876. ISSN: 0027-8424.

AU Le Maout S; Welling P A; Brejon M; Olsen O; Merot J

AB The inwardly rectifying potassium channel Kir 2.3 is specifically **targeted** and expressed on the **basolateral membrane** of certain renal epithelial cells. In the present study, the structural basis for polarized **targeting** was elucidated. Deletion of a unique COOH-terminal domain produced channels that were mistargeted to the apical **membrane**, consistent with the removal of a **basolateral membrane**-sorting **signal**. By characterizing a series of progressively smaller truncation mutants, an essential **targeting signal** was defined (residues 431-442) within a domain that juxtaposes or overlaps with a type I PDZ binding motif (442). Fusion of the COOH-terminal structure onto CD4 was sufficient to change a random **membrane**-trafficking and expression pattern into a **basolateral membrane** one. Using metabolic labeling and pulse-chase and surface immunoprecipitation, we found that CD4-Kir2.3 COOH-terminal chimeras were rapidly and directly **targeted** to the **basolateral membrane**, consistent with a sorting **signal** that is processed in the biosynthetic pathway. Collectively, the data indicate that the **basolateral** sorting determinant in Kir 2.3 is composed of a unique arrangement of trafficking motifs, containing tandem, conceivably overlapping, biosynthetic **targeting** and PDZ-based **signals**. The previously unrecognized domain corresponds to a highly degenerate structure within the Kir channel family, raising the possibility that the extreme COOH terminus of Kir channels may differentially coordinate **membrane targeting** of different channel isoforms.

L32 ANSWER 9 OF 94 MEDLINE

AN 2001347526 MEDLINE

TI A dileucine motif **targets** E-cadherin to the basolateral cell surface in Madin-Darby canine kidney and LLC-PK1 epithelial cells.

SO JOURNAL OF BIOLOGICAL CHEMISTRY, (2001 Jun 22) 276 (25) 22565-72.
Journal code: HIV; 2985121R. ISSN: 0021-9258.

AU Miranda K C; Khromykh T; Christy P; Le T L; Gottardi C J; Yap A S; Stow J L; Teasdale R D

AB E-cadherin is a major adherens junction protein of epithelial cells, with a central role in cell-cell adhesion and cell polarity. Newly synthesized E-cadherin is **targeted** to the **basolateral** cell surface. We analyzed **targeting** information in the cytoplasmic tail of E-cadherin by utilizing chimeras of E-cadherin fused to the ectodomain of the interleukin-2alpha (IL-2alpha) receptor expressed in Madin-Darby canine kidney and LLC-PK(1) epithelial cells. Chimeras containing the full-length or **membrane**-proximal half of the E-cadherin cytoplasmic tail were correctly **targeted** to the **basolateral** domain. **Sequence** analysis of the **membrane**-proximal tail region revealed the presence of a highly conserved dileucine motif, which was analyzed as a putative **targeting signal** by mutagenesis. Elimination of this motif resulted in the loss of Tac/E-cadherin **basolateral** localization, pinpointing this dileucine **signal** as being both necessary and sufficient for **basolateral targeting** of E-cadherin. Truncation mutants unable to bind beta-catenin were correctly **targeted**, showing, contrary to current understanding, that beta-catenin is not required for **basolateral** trafficking. Our results also provide evidence that dileucine-mediated **targeting** is maintained in LLC-PK(1) cells despite the altered polarity of **basolateral** proteins with tyrosine-based **signals** in this cell line. These results provide the first direct insights into how E-cadherin is **targeted** to the **basolateral membrane**.

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FILE 'MEDLINE, AGRICOLA, CANCERLIT, SCISEARCH, CAPLUS, BIOSIS, MEDICONF'
ENTERED AT 18:10:52 ON 23 APR 2002
L1 56 S GPI SIGNAL SEQUENCE
L2 15 DUP REM L1 (41 DUPLICATES REMOVED)
L3 15 SORT L2 PY
L4 0 S L3 AND KIDNEY

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L3 ANSWER 5 OF 15 MEDLINE
AN 1998021059 MEDLINE
TI GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency.
SO JOURNAL OF BIOCHEMISTRY, (1997 Aug) 122 (2) 251-7. Ref: 50
Journal code: HIF; 0376600. ISSN: 0021-924X.
AU Kinoshita T; Ohishi K; Takeda J
AB Protein GPI anchors are ubiquitous in eukaryotic cells. More than 50 mammalian proteins are anchored to the membrane via GPI. GPI anchoring is a posttranslational modification occurring in the endoplasmic reticulum where preassembled GPI anchor precursors are transferred to proteins bearing a C-terminal **GPI signal sequence**.
The GPI anchor precursors are synthesized in the endoplasmic reticulum by sequential addition of sugar and other components to phosphatidylinositol. More than ten genes participate in this biosynthetic pathway, eleven of the mammalian genes having been cloned by means of complementation of mutant cells that are defective in this pathway or based on sequence homology to previously cloned yeast counterparts. A somatic mutation in one of those genes, PIG-A, involved in the first reaction step, is responsible for the hemolytic disease, paroxysmal nocturnal hemoglobinuria.

L3 ANSWER 12 OF 15 MEDLINE
AN 2001227367 MEDLINE
TI Removal of the circumsporozoite protein (CSP) glycosylphosphatidylinositol signal sequence from a CSP DNA vaccine enhances induction of CSP-specific Th2 type immune responses and improvesprotection against malaria infection.
SO EUROPEAN JOURNAL OF IMMUNOLOGY, (2001 Mar) 31 (3) 692-8.
Journal code: EN5; 1273201. ISSN: 0014-2980.
AU Scheiblhofer S; Chen D; Weiss R; Khan F; Mostbock S; Fegeding K; Leitner W W; Thalhamer J; Lyon J A
AB The C terminus of the circumsporozoite protein (CSP) is anchored to the parasite cell membrane by a glycosylphosphatidylinositol (GPI) glycolipid. This **GPI signal sequence** functions poorly in heterologous eukaryotic cells, causing CSP retention within internal cell organelles during genetic immunization. Cellular location of antigen has quantitative and qualitative effects on immune responses induced by genetic immunization. Removal of the **GPI signal sequence** had a profound effect on induction and efficacy of CSP-specific immune response after genetic immunization of BALB/c mice with a gene gun. The CSP produced from the plasmid lacking the GPI anchor signal sequence (CSP-A) was secreted and soluble, but that produced by the CSP+A plasmid was not. The CSP-A plasmid induced a highly polarized Th2 type response, in which the CSP-specific IgG antibody titer was three- to fourfold higher, and the protective effect was significantly greater than that induced by the CSP+A plasmid. Thus, these two physical forms of CSP induced quantitatively and qualitatively different immune responses that also differed in protective efficacy. Engineering plasmid constructs for proper cellular localization of gene products is a primary consideration for the preparation of optimally efficacious DNA vaccines.

L3 ANSWER 15 OF 15 MEDLINE
AN 2002128794 IN-PROCESS
TI Mutational analysis of the variant surface glycoprotein GPI-anchor signal sequence in Trypanosoma brucei.
SO JOURNAL OF CELL SCIENCE, (2002 Feb 15) 115 (Pt 4) 805-16.
Journal code: 0052457. ISSN: 0021-9533.

AU Bohme Ulrike; Cross George A M

AB The variant surface glycoproteins (VSG) of Trypanosoma brucei are anchored to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. All GPI-anchored proteins are synthesized with a C-terminal signal sequence, which is replaced by a GPI-anchor in a rapid post-translational transamidation reaction. VSG **GPI signal sequences** are extraordinarily conserved. They contain either 23 or 17 amino acids, a difference that distinguishes the two major VSG classes, and consist of a spacer sequence followed by a more hydrophobic region. The omega amino acid, to which GPI is transferred, is either Ser, Asp or Asn, the omega+2 amino acid is always Ser, and the omega+7 amino acid is almost always Lys. In order to determine whether this high conservation is necessary for GPI anchoring, we introduced several mutations into the signal peptide. Surprisingly, changing the most conserved amino acids, at positions omega+1, omega+2 and omega+7, had no detectable effect on the efficiency of GPI-anchoring or on protein abundance. Several more extensive changes also had no discernable impact on GPI-anchoring. Deleting the entire 23 amino-acid signal sequence or the 15 amino-acid hydrophobic region generated proteins that were not anchored. Instead of being secreted, these truncated proteins accumulated in the endoplasmic reticulum prior to lysosomal degradation. Replacing the **GPI signal sequence** with a proven cell-surface membrane-spanning domain reduced expression by about 99% and resulted not in cell surface expression but in accumulation close to the flagellar pocket and in non-lysosomal compartments. These results indicate that the high conservation of the VSG **GPI signal sequence** is not necessary for efficient expression and GPI attachment. Instead, the GPI anchor is essential for surface expression of VSG. However, because the VSG is a major virulence factor, it is possible that small changes in the efficiency of GPI anchoring, undetectable in our experiments, might have influenced the evolution of VSG **GPI signal sequences**.

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(57) Abstract

The invention relates to recombinant DNA constructs, a method for producing a recombinant biologically active protein *in vivo* in the urine of a non-human mammal using a kidney-specific promoter, such as the uromodulin promoter, and the transgenic non-human mammals that serve as urine-based bioreactors for protein production.

Urothelial function reconsidered: A role in urinary protein secretion

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Mammalian bladder epithelium functions as an effective permeability barrier. We demonstrate here that this epithelium can also function as a secretory tissue directly involved in modifying urinary protein composition. Our data indicate that normal bovine urothelium synthesizes, as its major differentiation products, two well-known proteases: tissue-type plasminogen activator and urokinase, as well as a serine protease inhibitor, PP5. Moreover, we demonstrate that the urothelium secretes these proteins in a polarized fashion into the urine via a cAMP- and calcium-regulated pathway. Urinary plasminogen activators of ruminants are therefore urothelium derived rather then kidney derived as in some other species; this heterogeneity may have evolved in response to different physiological or dietary factors. In conjunction with our recent finding that transgenic mouse urothelium can secrete ectopically expressed human growth hormone into the urine, our data establish that normal mammalian urothelium can function not only as a permeability barrier but also as a secretor of urinary proteins that can play physiological or pathological roles in the urinary tract.

Mammalian urothelium lines the lower urinary tract and has evolved into a highly effective permeability barrier that can maintain steep chemical gradients (1). Thus, the permeability of urothelium to water, urea, ammonia, and protons is among the lowest in biological membranes (2). To perform this barrier function, urothelium makes extensive tight junctions and elaborates a highly specialized apical surface, which is almost completely covered with 0.2–0.5-μm rigid-appearing plaques (1, 3). These urothelial plaques consist of two-dimensional crystalline, hexagonal arrays of 16-nm particles composed of uroplakins, which represent major differentiation products of mammalian urothelium (4–9). Consistent with such a highly structured apical surface, which may seem incompatible with a secretory function, normal urothelium is not known to affect the composition of urine proteins, which are synthesized either by the liver (e.g., the major urinary protein complex of rodents), gaining access to the urine by glomerular filtration (10), or by the kidneys [uromodulin and epidermal growth factor (11, 12)]. We show here, however, that normal bovine urothelium serves as the main source of a major class of soluble, urine proteins including urokinase, tissue-type plasminogen activator, and a potent serine protease inhibitor, PP5. In conjunction with our recent finding that transgenic mouse urothelium can secrete human growth hormone into the urine (13), these results change our concept about the function of mammalian urothelium, which must now be considered not just as a permeability barrier but also as a possible source of urine proteins that may play important physiological or pathological roles in lower urinary tract.

Materials and Meth ds

Subtractive cDNA Library. Bovine urothelial cDNAs were used as the "tester" (20 ng), whereas those from nonurothelial tissues were used as the "driver" (600 ng) to generate a urothelium-specific cDNA library by suppression-subtractive hybridization (14) by using a subtractive kit (CLONTECH). Southern blot was done by separating the PCR products on a 2% agarose gel, transferring the DNA to a nylon membrane, and hybridizing with a ^{32}P-labeled cDNA probe. Northern blot was done by using 20 μg of total RNA. Probes were labeled with the Multiprime DNA-labeling system (Amersham Pharmacia). The PCR-amplified cDNA products of the subtractive library were packaged into PCRII vector by using a T/A cloning kit (Invitrogen) and were cloned. Plasmid DNA was isolated by using QIAprep spin miniprep kit (Qiagen, Chatsworth, CA). DNA sequencing was performed by dideoxynucleotide chain termination method (15) by using a T7 DNA sequence kit (Amersham Pharmacia).

Antibodies and Other Reagents. The sources of antibodies and other reagents are as follows: a rabbit antiserum to total uroplakins (4); mouse monoclonal antibodies AE1 and AE3 to keratins (16); a 52-kDa human high molecular mass urokinase (uPA), a 65-kDa 2-chain human tissue-type plasminogen activator (tPA), a rabbit antiserum to mouse uPA, and mouse monoclonal antibodies to human tPA and uPA (American Diagnostica, Greenwich, CT); a rabbit antiserum to rat PP5 (a generous gift from W. Kisiel of the University of New Mexico); affinity-purified horseradish peroxidase-conjugated goat antibodies to mouse and rabbit IgG (ICN); and bovine plasminogen, thrombin, fibrinogen, and brefeldin A (Sigma).

Immunohistochemistry. Tissue sections were incubated sequentially with 1% hydrogen peroxide in methanol to block the endogenous peroxidase, 2% goat serum, primary antibodies in 2% goat serum at 4°C overnight, and finally specific horseradish peroxidase-conjugated secondary antibodies. The sections were counterstained with hematoxylin, mounted in glycerin gelatin, and observed with a Zeiss microscope.

Cell and Organ Culture of Bovine Urothelium. Bovine urothelial cells were cultured at 37°C in the presence of mitomycin C-treated NIH 3T3 feeder cells in DMEM containing 15% FCS/0.5 μg/ml hydrocortisone/5 ng/ml cholera toxin/5 μg/ml insulin/15 ng/ml epidermal growth factor (17). 3T3 feeder cells and any contaminating fibroblasts were removed by using 0.01% EDTA in PBS when urothelial cells reached ≈70% confluence. Conditioned media were prepared by incubating the cells with fresh DMEM (without serum) for 24 h, collecting the medium, and centrifuging it at 3,000 × g for 10 min to remove cell debris. Total cell lysates were prepared by dissolving the cells in a solution containing 0.1 M Tris·HCl (pH 7.4), 0.1% SDS, 0.1% Nonidet P-40. In some experiments, cells (5 days postconfluent and incubated overnight with growth medium without cholera toxin) were washed twice with a release buffer (10 mM Hepes, pH

Abbreviations: tPA, tissue-type plasminogen activator; uPA, urokinase; PP5, placenta protein 5.

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7.0/150 mM NaCl/5 mM KCl) and incubated at 37°C for 30 min in the same buffer with or without 1.0 mM 8-BrcAMP or 1.0 mM calcium ionophore A23187. For assessment of polarized secretion, urothelial cells were plated in a (6-well) Transwell plate on 24-mm Nucleopore filters (0.4-mm pore size; Costar) that had been soaked in a serum-containing medium for 1 h. The cells were incubated with a serum-free medium for 24 h before the media were collected from the apical and basal compartments of the Transwell plate.

For organ culture, bladder mucosa was peeled from a fresh bovine bladder. The isolated mucosa consisting of the intact urothelium and some underlying stromal layers (total thickness ≈2 mm; surface area ≈5 × 5 cm) was rinsed with DMEM and sandwiched, urothelial side up, between two plastic (24-well) plates specially designed so that both the urothelial surface and the underlying mesenchymal tissue can be completely soaked in a serum-free DMEM medium.

Immunoblot and Fibrin Zymography. Proteins were separated by SDS/PAGE on a 10% polyacrylamide gel (acrylamide/bisacrylamide ratio, 120:1) and electrophoretically transferred to nitrocellulose membrane. After incubation with 2% nonfat milk in PBS, the membrane was incubated with primary and horseradish peroxidase-conjugated secondary antibodies, treated with an enhanced chemiluminescence kit (Pierce), and exposed to Fuji x-ray film.

For zymography, an SDS gel was rinsed with 2.5% Triton X-100 (2 × 30 min) and PBS (10 min), laid carefully onto a fibrin–agarose indicator gel, sealed with plastic wrap, and incubated at 37°C. For *in situ* zymography, 0.5 ml of indicator gel solution was put on a 10-μm (unfixed and air-dried) frozen tissue section and immediately covered by a 25 × 25-mm glass coverslip. The section was incubated at 37°C in a humid chamber. In some experiments, the sections were preincubated with antibodies against tPA and/or uPA at room temperature for 30 min and washed with PBS 2 × 5 min before applying the indicator gel (18, 19). The fibrin–agarose indicator gel contained 5 μg/ml bovine plasminogen, 0.06 units/ml bovine thrombin, 1.8 mg/ml bovine fibrinogen, 3.2% nonfat milk, 1% agarose (20).

Results and Discussion

uPA and tPA as Major Differentiation Products of Bovine Urothelium. To study the differentiation process of bladder epithelium, we generated a subtraction cDNA library of normal bovine urothelium by suppression subtractive hybridization (14). Common messages were eliminated by hybridizing the cDNA of bovine urothelium with those of 10 other tissues including kidney, lung, spleen, skeletal muscle, esophagus, stomach, intestine, colon, brain, and liver. This resulted in an estimated >1,000-fold enrichment of urothelial-specific cDNA, as evidenced by a >10-fold increase in the cDNA of uroplakin Ib, a well-established urothelial marker (7), and a >100-fold reduction in actin cDNA (Fig. 1 *A* and *B*). The most abundant cDNAs in this enriched library encoded uroplakins (32 of a total of 140 clones), thus validating the effectiveness of the subtraction technique. The next four most abundant cDNAs encoded PP5, a Kunitz-type serine protease inhibitor that can inhibit plasmin (27 clones) (21, 22); keratin 19, which is one of the major urothelial keratins (10 clones) (17, 23); tPA (9 clones); and uPA (6 clones). Northern blotting showed that all four genes were, like uroplakin genes, so highly expressed in bovine urothelium that they seemed urothelium "specific" (Fig. 1*C* and data not shown).

Given the current thought that tPA and uPA are synthesized and secreted into the urine mainly by the kidneys (24, 25), we were surprised to find that genes encoding plasminogen activators (PAs) and the PP5 protease inhibitor were so highly expressed in normal bovine urothelium. Immunoblot analysis

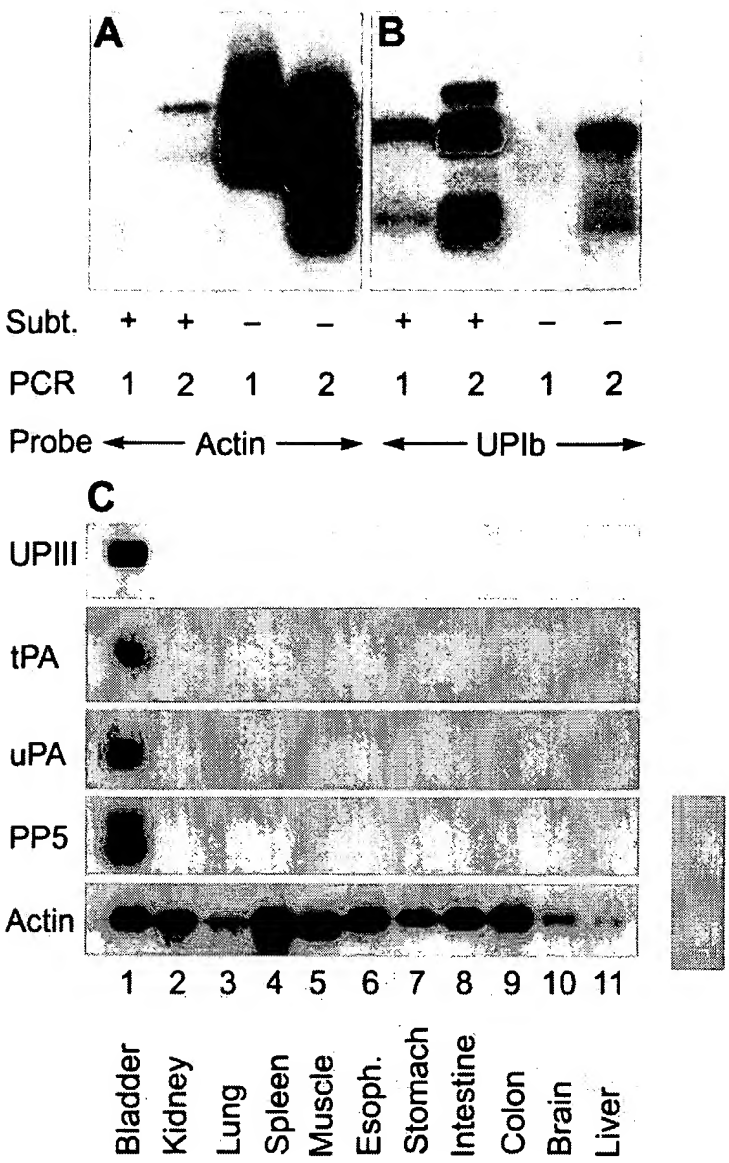

Fig. 1. Enrichment of urothelium-specific cDNAs in a bovine urothelial subtraction library. Common cDNAs in bovine urothelium were eliminated by suppression subtractive hybridization (14). (*A* and *B*) Southern blot analyses of the subtraction products. The *Pst*I-digested urothelial cDNA fragments before (−) or after (+) subtraction, which were PCR amplified once (marked 1) or twice (marked 2), were electrophoretically separated on an agarose gel and probed for (*A*) actin or (*B*) uroplakin Ib, a urothelial marker (7). Scanning of the autoradiograms showed a >100-fold reduction in actin and >10-fold enrichment in uroplakin cDNAs. (*C*) Northern blot analyses. Twenty micrograms of total RNAs from bovine urothelium and other tissues were fractionated by agarose gel electrophoresis and probed for uroplakin III, tPA, uPA, PP5 protease inhibitor, and actin (control).

confirmed, however, the presence of tPA (68 kDa), uPA (48 kDa), and PP5 (31 kDa) in the total protein extracts of (scraped) *in vivo* bovine urothelium (Fig. 2*A*). The two PAs were enzymatically active, as evidenced by their ability to activate plasminogen in zymography (20) (Fig. 2*B*). Immunolocalization showed that uPA and PP5 were associated with the upper cell layers (Fig. 2 *G* and *H*), whereas tPA was enriched in the superficial umbrella cells (Fig. 2*F*).

Urothelial Secretion of Proteases and Inhibitor. To examine in more detail the synthesis and secretion of PAs, we cultured bovine

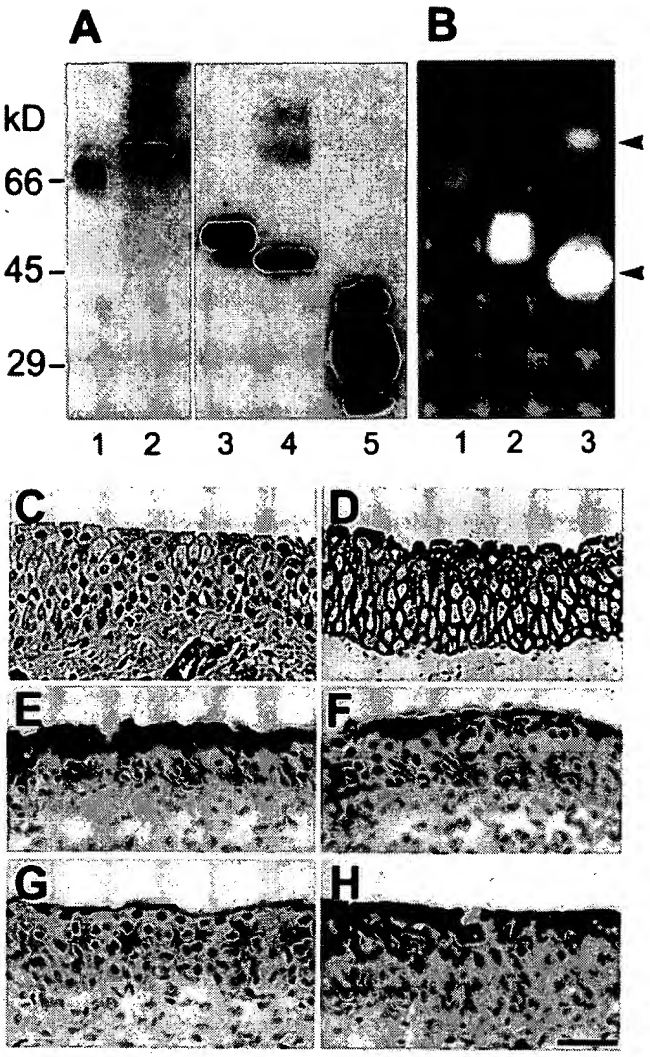

Fig. 2. Detection of PAs and PP5 in bovine urothelium. (*A*) Immunoblot analysis. Total bovine urothelial proteins (10 μg; lanes 2, 4, and 5), recombinant human (rh) tPA (66 kDa, 0.2 μg; lane 1), or rh-uPA (52 kDa, 0.2 μg; lane 3) produced in bacteria were separated by SDS/PAGE and immunoblotted with antibodies against tPA (1 and 2), uPA (3 and 4), and PP5 (5). Note the detection of bovine tPA (68 kDa), uPA (48 kDa), and PP5 (27, 31, and 35 kDa) in bovine urothelial extracts; the three PP5 bands reflect different degrees of glycosylation (ref. 50 and data not shown). The sizes of the human and bovine PAs are known to be slightly different. (*B*) Detection of fibrinolytic activities of rh-tPA (lane 1, 0.1 μg), rh-uPA (land 2, 0.1 μg), and a total bovine urothelial extract (lane 3, 10 μg) by zymography. The SDS gel was overlaid with a fibrin-containing indicator gel, incubated at 37°C for 1–3 h, followed by Coomassie blue staining of the indicator gel. Note the bovine tPA (68-kDa) and uPA (48-kDa) bands (arrowheads). (*C–H*) Immunolocalization of the PAs and PP5. Paraffin sections (5 μm) of bovine bladder were stained with hematoxylin and eosin (*C*), AE1 and AE3 antibodies to keratins (*D*) (16, 51), rabbit antitotal uroplakins (*E*) (4), mouse anti-tPA (*F*), rabbit anti-uPA (*G*), and rabbit anti-PP5 (*H*) (50). Note the staining of superficial cells by anti-tPA and anti-PP5 and the suprabasal cells by anti-uPA. (*C–H*) Same magnification. (Bar = 50 μm.)

urothelial cells in the presence of 3T3 feeder cells. Under this condition, bovine urothelial cells formed stratified colonies and expressed uroplakins in the suprabasal cells (Fig. 3*A*; refs. 17 and 26). These cells also expressed tPA (Fig. 3*B*) and uPA (Fig. 3*C*) in a differentiation-dependent manner, consistent with the immunolocalization data (Fig. 2; see ref. 27). Although only ≈5% of the PA activities were secreted into the culture medium in 30 min, this secretion could be rapidly up-regulated to 25–35% by 8-Br-cAMP or calcium ionophore A23187 (Fig. 3*D*; also see *E* and *F*). This result is consistent with an earlier finding that tPA secretion by cultured human endothelial cells can be stimulated by cAMP (28, 29) and suggests that the secretion of PA by urothelium occurs via a regulated pathway, which may enable the enzymes to be acutely released in response to certain physiological stimuli such as wounding. But for this secretion to be functional in the urine, it must occur apically. Previous studies indicate that the polarity of PA secretion by epithelia is cell type dependent. Thus, human intestinal epithelial $CaCO_2$ cells and HeLa cells secrete uPA basolaterally; canine kidney MDCK cells secrete uPA apically; and rat FRT thyroid cells secrete tPA apically (30, 31). That urothelial cells secreted both tPA and uPA in a polarized fashion mainly via their apical surface was demonstrated by the fact that >90% of the extracellular PAs were found in the apical compartment when the cells were grown on a Nucleopore filter in a dual compartment culture system (Fig. 3 *E* and *F*).

Because the physiological state of cultured cells can be very different from the *in vivo* tissues, we studied the secretion of PAs by normal bovine urothelium maintained in short-term organ culture. A piece of fresh bladder mucosa was sandwiched between two plastic plates with holes containing tissue culture media. A tPA/uPA activity ratio of ≈1/8 was observed in bovine urine (Fig. 3*G*, lane 2); a similar ratio was observed in normal bovine urothelial extract (lane 1) as well as in a medium that had been conditioned by organ-cultured bovine urothelium (lane 3). This apical secretion of the PAs was blocked by brefeldin A, an inhibitor of the secretory process (lane 4) (32), proving that the PAs in the culture medium were actively secreted rather than leaked from lysed cells. The PA concentrations in the cell and organ culture-conditioned media (50–200 μg of tPA and 100–150 μg of uPA/liter) were roughly equivalent to those of bovine urine (≈250 μg of tPA and ≈500 μg of uPA/liter), suggesting that urothelial secretion can account for most of the urinary PAs (Table 1). Finally, we showed that PP5, which was present in the urine (Fig. 3*H*, lane 2), was also secreted by organ-cultured urothelium into the medium (Fig. 3*H*, lane 3).

The synthesis of PAs and PP5 by cultured urothelial cells differed significantly from the organ-cultured urothelium. Thus cultured urothelial cells synthesized and secreted relatively more tPA (with a tPA/uPA activity ratio of 1:3 vs. 1:8 in organ culture). Such an altered tPA/uPA ratio may be caused by different tissue culture conditions and the accompanied changes in the differentiation state of the urothelial cells. In addition, the cultured urothelial cells completely suppressed the synthesis of PP5 (Fig. 3*H*, lanes 5 and 6). Because, as we suggested earlier (17), cultured urothelial cells mimic a regenerative urothelium, the down-regulation of the protease inhibitor in such a "healing" urothelium may lead to the enhancement of the proteolytic activities that are frequently associated with *in vivo* wound healing and tissue remodeling.

Species Variation in the Site of PA Synthesis. To determine the distribution of PAs in the bovine urinary tract, we performed *in situ* zymography by using unfixed cryo-tissue sections. As expected, bovine urothelium showed a high fibrinolytic activity as evidenced by its ability to produce a clear zone in an opaque fibrin-containing indicator gel (Fig. 4*A*); this activity could be blocked by a combination of antibodies to tPA and uPA (Fig. 4 *B–D*), indicating that these two enzymes could account for most of the detected fibrinolysis. Very little fibrinolytic activity was detected in bovine kidney sections even after prolonged incubation (Fig. 4 *E* and *F*). Similar results were obtained with the bladder and kidney tissues of sheep, another ruminant (data not shown). This situation is quite different from those of the mouse and human urinary tracts. Mouse urothelium had a low fibrinolytic activity (Fig. 4 *G* and *H*), whereas its kidneys

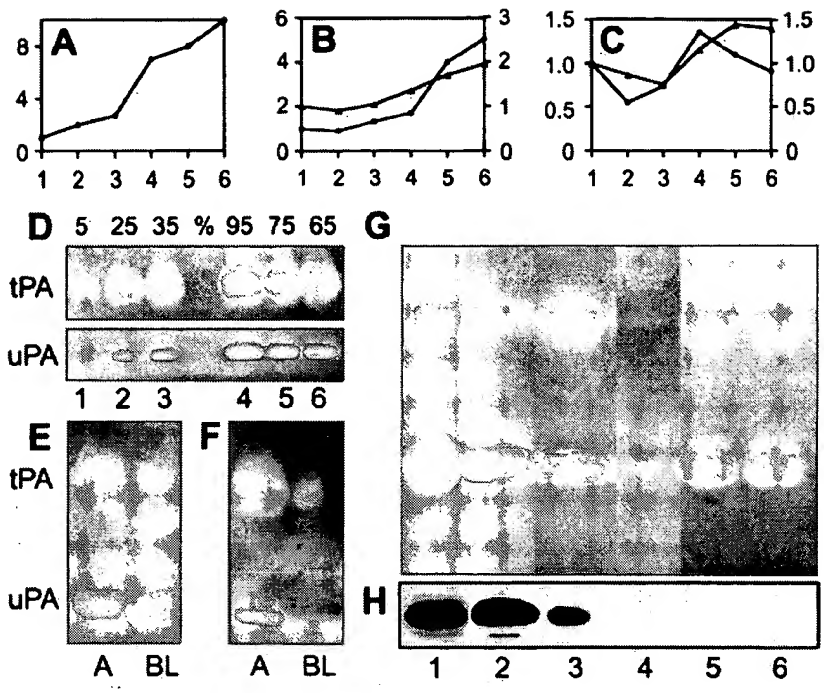

Fig. 3. Differentiation-dependent expression and apical secretion of plasminogen activators by cell- and organ-cultured bovine urothelium. (*A–C*) mRNA levels (relative to glyceraldehyde-3-phosphate dehydrogenase as assessed by Northern blot, ●) and PA activities (zymography, ▲) of uroplakin III (*A*), tPA (*B*), and uPA (*C*) in cultured bovine urothelial cells (17). Cells were harvested at 25% confluence (lane 1), 50% (lane 2), 100% (lane 3), 3 days postconfluence (pc) (lane 4), 6 days pc (lane 5), and 9 days pc (lane 6). Loading was normalized on the basis of cell numbers. Note the differentiation-dependent expression of UPIII and tPA and, less strikingly, of uPA. (*D*) Stimulation of PA secretion. Cultured bovine urothelial cells were treated with a control medium (lanes 1 and 4), 1 μM calcium ionophore A23187 (lanes 2 and 5), and 1 mM 8-Br-cAMP (lanes 3 and 6). Numbers above denote the percentages of PA activities in conditioned media (lanes 1–3) vs. cell lysates (lanes 4–6). (*E*) Polarized secretion of PAs. Bovine urothelial cells were cultured on a Nucleopore filter (Transwell, 0.4-mm pore size; Costar). A and BL denote the apical and basal-lateral compartments, respectively. The media were collected after 24 h in a control medium (*E*) or, after 15 min, in the presence of 1 mM of cAMP (*F*). Note that a great majority of the PAs were secreted apically and that the polarity was not affected by cAMP stimulation. (*G*) Secretion of tPA and uPA by organ-cultured bovine bladder mucosa into the overlying culture medium. Samples were 20 μg of total protein extract of *in vivo* bovine urothelium (lane 1), 15 μl of fresh bovine urine (lane 2); 20 μl of a medium that had been conditioned by organ-cultured bovine urothelium for 3 h (lane 3); 20 μl of medium conditioned in the presence of 5 μg/ml brefeldin A, a secretory inhibitor (lane 4) (32); 5 μg of total protein extract of 5-day postconfluent cell-cultured bovine urothelium (lane 5); and 20 μl of the culture medium that had been conditioned for 24 h by 5-day postconfluent cells (lane 6). Note the secretion of tPA and uPA by organ-cultured urothelium and its inhibition by BFA. (*H*) Detection of PP5 in the same samples as in *G* by immunoblotting. Note the detection of PP5 in urothelium, fresh urine, and in a medium conditioned by organ-cultured bovine urothelium; also note the absence of PP5 in cultured urothelial cells.

had very high activities (Fig. 4 *I* and *J*), consistent with previous *in situ* hybridization data (33–35). Similarly, the reported PA content of normal human urothelium [60 ng tPA/mg and 2 ng uPA/mg total urothelial proteins (27, 36)] was several hundred-fold lower than that of normal bovine urothelium (Table 1). Together, these results indicate that although mouse and human urinary PAs are mainly kidney derived (Fig. 4 *G–J*; refs. 24 and 33), ruminant PAs are primarily urothelium-derived (Fig. 4 *A–F*; summarized in Fig. 4*K*).

Possible Functions of Urinary PAs. Thus, bovine urothelium synthesizes and secretes into the urine large amounts of uPA and tPA as well as, importantly, a potent protease inhibitor: PP5. Also known as tissue factor pathway inhibitor-2, PP5 is a serine protease inhibitor consisting of three tandemly arranged Kunitz-type domains (21). PP5 can directly or indirectly inhibit kallikrein, plasmin, and PAs (22). The coexistence of PAs with PP5 in the urine suggests that their activities must be tightly regulated. That urine of all animal species examined contained

Table 1. Concentrations of plasminogen activators in bovine urine, urothelia-conditioned media, and urothelial cells, as calculated from the zymography data

		Conditioned media, μg/liter*		Urothelium, μg/mg cellular protein	
	Urine, μg/liter	Organ culture	Cell culture	*In vivo*	Cell culture
uPA	500†	100	150	10	8
tPA	250	50	200	5	15

*Because bovine bladder has an average surface area of ≅200 cm^2, in contact with ≅5 liters of urine per day, these numbers were adjusted to be equivalent to media exposed to cultured urothelial cells at 37°C for 24 h, at a ratio of 25 ml/cm^2 surface area of cells.

†Similar concentrations of uPA were found in human (100–1,000 μg/liter) (ref. 38 and data not shown) and mouse urine (≅1,800 μg/liter) (49).

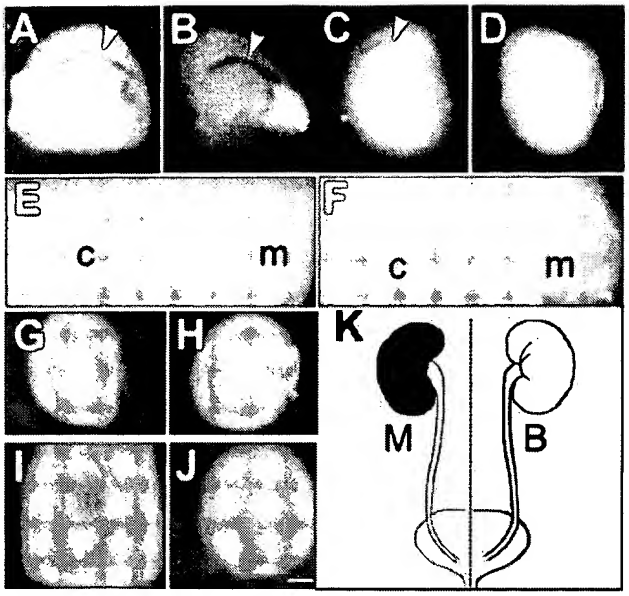

Fig. 4. Distribution of plasminogen activator activities in bovine vs. mouse urinary tract. Frozen tissue sections (10 μm) were air dried and preincubated at 37°C for 30 min with PBS (*A*), anti-tPA (*B*), anti-uPA (*C*), and a mixture of anti-tPA and anti-uPA (*D*) before they were overlaid with an (opaque) fibrin-containing indicator gel and incubated at 37°C for 30 min. Fibrinolysis by plasminogen activator results in a clear zone. (*A–F*) Sections of bovine bladder (*A–D*) or kidney (*E, F*). Note the strong fibrinolytic activities in the urothelial zone (arrowheads) and their complete inhibition by a mixture of anti-tPA and anti-uPA. The bovine kidney sections were incubated with the indicator gel for a prolonged period of 3 h (*E*) and 8 h (*F*) to show the barely detectable PA activities. (*G–J*) Sections of mouse bladder (*G* and *H*) or kidney (*I* and *J*) that were overlaid with indicator gels with (*G* and *I*) or without (*H* and *J*) plasminogen. Note the strong fibrinolytic activities in mouse kidney (*I*) and their absence in mouse bladder (*G*). (*K*) Site of PA production (marked red) in the urinary tracts of mouse (M) and bovine (B). c, cortex; m, medulla. All pictures are of the same magnification. (Bar = 5 mm.)

significant amounts of fibrinolytic enzymes (Fig. 3; refs. 20 and 37) strongly suggests that these enzymes play important functional role(s) in the urinary tract. These roles have not yet been clearly defined, but they may include the following. First, they may be involved in extracellular proteolysis to prevent or circumvent the obstruction of the urinary tract because of protein precipitation or fibrin clot formation (33). Second, urinary stone formation or urolithiasis may involve the precipitation of a matrix of mucoproteins followed by crystallization of minerals onto this matrix. Urinary proteases may prevent the formation of protein nucleation/matrix (38). Third, urothelial desquamation is an important defense mechanism against bacterial attachment (39, 40), and PAs and their inhibitors seem to play a role in regulating desquamation (41). Fourth, PAs and their inhibitors may play a role in urothelial migration and tissue remodeling, as has been shown to occur in many cell types (42–44). Finally, urinary PAs and kallikrein, another urinary protease that can activate pro-uPA (45), can digest and inactivate the sodium channels on the urothelial surface, thereby playing a role in regulating sodium transport (46). That ruminant urinary PAs are synthesized by the urothelium, instead of the usual kidneys, suggests that PAs are not needed in the ruminant kidneys, possibly because of different dietary or physiological factors.

Concluding Remarks. Although mammalian urothelium is thought to function mainly as an exceptionally effective permeability barrier that can withstand repeated stretching, we show here that bovine urothelium can, in addition, serve as a major supplier for a class of soluble urinary proteins. This establishes that bovine urothelium can actively modify the protein composition of the urine; that this polarized secretory process can be regulated by cAMP and calcium; and that urothelium can perhaps serve as a better transgenic "bioreactor" in secreting recombinant human proteins into the urine than previously thought feasible, at least when ruminants are used in such "biofarm" applications (13). That the secretory activity of urothelium is not limited to the ruminants is demonstrated by our recent finding that mouse urothelium ectopically expressing human growth hormone can secrete this protein into the urine (13). Taken together, these results indicate that mammalian urothelia in general can secrete urine proteins that may play physiological or pathological roles in the lower urinary tract (47). Additional data are needed to understand how proteins are secreted through the plaque-covered apical urothelial surface, e.g., whether the secretion occurs selectively at the interplaque or the so-called "hinge" areas (3, 48).

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